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SUMMARY

Chapter I: INTRODUCTION

In this investigation the development of the rat cerebellum from 0 - 30 days after birth is studied morphologically, by means of enzyme histochemistry and electron microscopy. Enzyme histochemistry and electron microscopy were chosen because changes in enzyme content or enzyme pattern and ultrastructural alterations are major features in processes of growth and especially differentiation. The cerebellum was chosen as a relatively clearly arranged part of the central nervous system. In the rat it is still rudimentary at birth; maturation completes in about 4 weeks' time.

In view of the complexity of developmental processes we feel that experimental studies are needed to assess the relative importance of different factors of possibly causative significance. Experimental procedures, in order to yield results relevant to the problems of normal development, must satisfy several conditions. They must be rapidly effective, reproducible and selective and must not give rise to tissue reactions disturbing development in a non-specific way. Irradiation with X-rays promised sufficiently to satisfy these conditions.

Chapter II: MATERIAL AND METHODS

Cerebellar development was studied in albino rats in sagittal sections through the vermis.

Paraffin sections were stained with a variety of methods. For Golgi preparations blocks were impregnated and processed according to MOLINER (1958). Several enzymes were studied histochemically in cryostat sections. The list of enzymes and the methods used are given on page 5. For electron microscopy animals were perfused with a solution containing 4 % paraformaldehyde and $\frac{1}{2}$ % glutaraldehyde in a veronalacetate buffer (pH 7.38). Small pieces of dorsal vermis were postfixed in 1 % OsO₄, dehydrated and embedded in Epon. Thin sections were stained with uranylacetate and leadcitrate. Irradiation was carried

out with a Siemens Dermopan. Animals were irradiated at the cerebellar area with $\pm 1000\text{r}$ (65 r/sec.; 43 kV; 25 mA; 0.6 mm Al-filter) through a tube with a diameter of 1 cm. The focus-skin distance was 5 cm.

Chapter III:

Some introductory data are given on the anatomy, histology and early development of the rat cerebellum.

Chapter IV: THE STUDY OF THE NORMAL DEVELOPMENT

The development of the cerebellum is described as studied in paraffin sections and Golgi preparations, enzyme histochemically and electronmicroscopically, in 0, 4, 8, 12, 16, 20 and 30 day-old rats.

At birth the **external granular layer** is present throughout the vermis. In the first stages the layer increases in thickness. At 8 days a distinction between an external matrix and a mantle layer is evident; mitoses are confined to the matrix. From 16 days onward the layer gradually disappears and it is no longer present at 30 days. The cells in this layer have strongly pyroninophilic cytoplasm, containing many polysomes, some mitochondria and Golgi complexes (fig. 72). They show moderate dehydrogenase and TPPase activity. Neither enzyme histochemically nor electronmicroscopically any differences are seen between cells in the matrix and in the mantle layer at 8, 12 and 16 days. The cerebellar surface in all stages is covered by a continuous basement membrane. The surface itself in early stages is formed by external granular cells. Later on these are gradually replaced by glial cell processes (cf. fig. 72 and 73).

The **molecular layer** is indistinct at birth, but takes shape at 4 and 8 days (figs. 33, 35, 39, 41, 47). At 8 days Purkinjecell-dendrites, parallel fibres, migrating neuroblasts, some neurones and blood vessels are present in this layer. From 12 days onward the peripheral part of the Purkinjecell-dendrites, the finer branches and spiny branchelets, develop (figs. 27, 28, 69). The number of parallel fibres increases. The extracellular space decreases while the compartment occupied by glial cell processes increases (figs. 74, 75). In later stages glial cell processes cover all other structures (Purkinjecell-dendrites, groups of parallel fibres) except for sites of synaptic contact. Basket cells appear at the end of the first week, stellate cells develop during the second week. The last cells to be formed are glial cells. In early stages there is little enzyme activity. At 8 and 12 days it is present mainly in Purkinjecell-dendrites. From 12 days onward diffuse activity of dehydrogenases, ATPase and - very strikingly - AMPase increases, spreading towards the brain surface (figs. 5-7, 21-23). Basket and stellate cells show TPPase, AcPase, α NE and IE activity.

Purkinjecells are difficult to identify at birth. At 4 and 8 days a row of cells is formed at the outside of the prospective internal granular layer (figs. 33, 35, 39, 41, 47). In the early stages the nucleus lies at the base of the cells. The cytoplasm shows high dehydrogenase, TPPase and AcPase activity, the latter two in a distinct part of the cytoplasm only (figs. 8, 9-11, 15-17). The cell border is irregular with many lateral extrusions, some showing synapses at 8 days (figs. 8, 76, 77, 81). Between 8 and 12 days remarkable changes occur in the Purkinje cells. At 12 days the nucleus is in the centre of the cytoplasm. Mitochondria, Golgi complexes, Nissl substance, ribosomes and dehydrogenase, TPPase and AcPase activity are distributed much more evenly through the cell (figs. 12, 14, 18-20, 51, 78, 79). Hereafter little change occurs in the perikaryon:

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the further development of the cells consists of growth of the peripheral dendrites.

Bergmann glialcells are even more difficult to identify in early stages. Golgi preparations however show their presence already at 0 days. Actually, we think that most cells scattered around and underneath the Purkinje cells at 0 and 4 days are Bergmann cells or their immediate precursors. In these stages the cells show very high α NE activity. At 8 days these cells are located in the outer part of the developing internal granular layer (some showing mitotic figures), whereas at 12 days they have taken their adult position around Purkinje cells (fig. 51). From 8 days onward they show high activity of NADP-linked dehydrogenases (ICDH, G6PDH - fig. 65). Electronmicroscopically the nucleus shows finely distributed chromatin; the cytoplasm is clear, with few ribosomes or endoplasmic reticulum, some mitochondria and Golgi complexes (figs. 78, 80); incidentally filaments, centrioles or cilia are present. The growth of the processes has already been discussed for the molecular layer. From 8 days onward also the cytoplasm of the Purkinje cells, apart from synaptic sites, is covered by glialcell cytoplasm.

The **internal granular layer** is present only from 8 days onward. As mentioned above, most cells present in the region of this layer in earlier stages seem to us to be Bergmann glialcells (this supposition is borne out by the effect of irradiation at 0, 2 and 4 days - after this no granule cells are seen at 30 days which implies that they are not present at the time of irradiation). At 8 days a few granule cells are present (fig. 47). By now the layer is rich in processes some showing synaptic contact; a large extracellular space is seen in electron micrographs (fig. 82). From 12 days onward cellularity increases and glomeruli develop (fig. 83). Glomerular formation starts in the archicerebellar areas at 12 days and is seen in other parts of the vermis at 16 days. The glomeruli show increasing activity of dehydrogenases (figs. 5-7), ATPase, AMPase, AcPase and N.ASD.E. AChE is strongest in archicerebellar glomeruli. *Pari passu* with the formation of glomeruli the number of synapses increases. In this layer, as in the molecular layer, a gradual decrease in the size of the extracellular space is paralleled by an increase in the glialcell-compartment. Golgicells are first seen at 6 days. In later stages these cells show remarkably high AcPase activity as compared with Purkinjecells.

The **white matter** shows mitotic activity at 8 and 12 days. At the time "active" glialcells occur, with a large nucleolus, pyroninophilic cytoplasm and high activity of dehydrogenases, TPPase and AcPase (figs. 5, 6, 13, 53). Astrocytes can be recognised from 8 days onward. In later stages active glialcells disappear while oligodendroglialcells make their appearance. Myelination progresses from 8 days onward. Even at 30 days early stages of myelination can still be seen electronmicroscopically.

The **central nuclei** were studied only incidentally. The development of the neurones shows a striking resemblance with that of Purkinjecells, as to the site of the nucleus, the development of pyroninophilia and enzyme activities. Development of these neurones is some days ahead of that of Purkinjecells. In later stages the neurones show a very high AcPase (fig. 14), α NE and IE activity in comparison with Purkinje (and even Golgi) cells; no such difference exists for TPPase activity, however.

Finally in chapter IV the development of **bloodvessels**, as seen enzymehistochemically, is described. There are great differences between the various enzymes showing vascular activity. The most complete "staining" is provided by the ATPase - reaction; early activity is seen in the central nuclei; at 12 and 16 days the white matter shows greater vascularity than the cortex; from 16 days onward the number of bloodvessels in the cortex strongly increases (figs. 24-26).

Some major features of the development of the molecular layer,

Purkinjecells, glomeruli and white matter are summarized in the tables on pages 21, 29, 31 and in the drawing on page 26.

Chapter V: THE INFLUENCE OF X-IRRADIATION ON CEREBELLAR DEVELOPMENT

Two series of irradiation-experiments are described in this chapter.

In the **first series** rats of different ages (0, 2, 4, 6, 8, 12, 16 and 20 days) were irradiated with 1000r, a dose which had been found to kill proliferating cells and cells in early stages of differentiation only. The immediate effect (celldeath) was studied after 6 hours, the ultimate effect on cerebellar development at 30 days, in paraffin sections.

6 hours after irradiation all cells of the external granular layer in all stages show severe degeneration (figs. 34, 36, 40, 42, 48, 52, 58). Scattered degenerating cells are seen in the other layers; these cells may very well represent dividing cells or cells in early stages of differentiation (such as migratory neuroblasts). In the white matter the effect of irradiation increases in the first stages, to decrease again from 16 days onward. At 8 and 12 days dividing cells and active glialcells seem to be particularly radiosensitive (fig. 54). Cells that appear well-differentiated at the time of irradiation (such as neurones of the central nuclei and also Purkinjecells in all stages) never show any sign of direct damage by X-rays.

The *ultimate effect* on cerebellar development varied with the time of irradiation. After irradiation at 0, 2 and 4 days the cerebella at 30 days are very small - no granule cells, basket and stellate cells having developed (figs. 37, 38). Purkinjecells are present, but distributed irregularly throughout the cortex (fig. 38). In paraffin sections Golgi and Bergmann cells are difficult to identify; this can be done by their enzyme activity as seen in the second series of experiments after irradiation at 2 days (figs. 64, 71). After irradiation at 6 and 8 days the cerebella are still markedly hypoplastic, especially in lobuli VI and VII (figs. 43, 49). This hypoplasia is due to non-development of most of the granule cells and *eo ipso* of the molecular layer. In this latter layer by now some neurones are seen (figs. 45, 50). Purkinjecells are more regularly distributed than after irradiation in earlier stages (figs. 45, 50). After irradiation at 12 days the cerebella at 30 days are still smaller than the normal ones (figs. 55, 56). After irradiation at 16 and 20 days differences between irradiated and normal cerebella at 30 days can no longer be seen (although they may still be found by quantitative analysis, which we did not carry out).

In the hypoplastic cerebella the white matter is also less well developed than normally. At 30 days no distinct differences in structure or cell-population can be seen in paraffin sections.

After irradiation in early stages, at 30 days some fibrosis of the leptomeninges occurs and some mast cells are present in the leptomeninges and perivascularly in the cerebellum (cf. OLSSON et al., 1965).

Fairly often some parts in otherwise affected cerebella show a completely normal development. We assume that these parts were not hit by the X-rays. In the boundary zones between normal and irradiated areas ectopic granule cells

are often present, either intermingled with Purkinjecells or in rows in the molecular layer - in these rows sometimes formation of glomeruli is suggested (fig. 46).

In the **second series** of experiments, 2 day-old rats were irradiated with 1000r and the consequent development of the cerebellum, especially of the Purkinjecells, was studied at 8, 16 and 30 days lightmicroscopically (including enzyme histochemistry) and electronmicroscopically.

Cerebellar development under these circumstances greatly differs from the normal. Apart from non-development of many celltypes (granule cells, basket and stellate cells) and failure of the cortical layers to constitute, the most striking abnormality in these cerebella is the poor development of the Purkinjecell-dendrites. The peripheral part of the dendritic tree, including the spiny branchlets, does not develop (cf. figs. 68 and 69). The cytoplasm of the Purkinjecells and the nucleus however show a completely normal pattern of differentiation as seen enzyme histochemically and electronmicroscopically.

In irradiated cerebellar cortex a uniformly high activity of some dehydrogenases (NAD-TR, LDH) is present (fig. 66). Many small cells show strikingly high ICDH activity (Bergmann cells - fig. 64). AcPase shows a distinction between neurones with low or moderate and high activity - Purkinjecells and Golgicells (fig. 71). By means of the AcPase-reaction the migration of Golgicells can be traced: at 8 days they are seen in the white matter, at 16 days in the inner part of the cortex and at 30 days they are scattered throughout the cortex. A striking positive correlation is seen between the presence of granule cells and AMPase activity (fig. 70). In the posterior vermis a conspicuously high subcortical AChE activity is present in all stages examined (fig. 67). This might be explained by the presence of AChE-positive mossy fibres lacking, under these conditions, their normal goal: the granule cell-dendrites. Abnormal axons are seen at 16 and 30 days: some are swollen, others contain regular tubular arrays that on transverse section show a honeycomb-like arrangement.

Chapter VI: DISCUSSION OF THE RESULTS

As the information collected is rather heterogeneous, an attempt of marshalling the data is made by discussing the results in the context of some categories.

a. CELLPROLIFERATION AND CELLMIGRATION

According to Fujita (*vide* FUJITA et al., 1966) three stages can be distinguished in the development of matrices in the central nervous system. In the first stage there is proliferation only, in the second stage some cells start differentiating into neuroblasts and in the last one glial cells develop. This conception can be usefully applied to cerebellar development, as has been shown by FUJITA himself (1967).

Processes of cellproliferation and migration in the developing cerebellum have been studied by means of labelling DNA-synthetizing cells with 3H-thymidine and autoradiography (MIALE and SIDMAN, 1961; FUJITA, ALTMAN and DAS, 1966; ALTMAN, 1966). The results of these studies are confirmed by the first series of irradiation experiments, in which proliferating cells and cells in early stages of differentiation were killed in different stages of cerebellar development. The results show from which matrix certain cells originate and (indirectly) the way in which they migrate. It can also be concluded at which time cells of a certain type have differentiated (*v.l.*) so far as to have lost their original high radiosensitivity. So a timetable of cerebellar development emerges, which is in good agreement with the results of experiments with radioactive thymidine.

celltype	matrix	time of differentiation as indicated by loss of radiosensitivity
neurones of the central nuclei	ventricular matrix	before birth
Purkinjecells	ventricular matrix	before birth
Golgicells	ventricular matrix	around birth
granulecells	external granular layer	\pm 4-16 days
basket cells	external granular layer	end of first week
stellate cells	external granular layer	second week

b. CELLDIFFERENTIATION

Celldifferentiation can be defined in many ways. In agreement with GROSS (1968) we used various criteria to assess differentiation: morphological features including the pattern of cellprocesses, the enzyme pattern and an "indirect" one, alteration in radiosensitivity (*v.s.*). These criteria are applied to the development of neurones and glial cells. In both groups striking differences exist between different cells. Many criteria of differentiation can be applied to Purkinjecells, differentiation of granule cells however is evident only from the growth of cellprocesses and from the decrease in radiosensitivity. Astrocytes show little change during development whereas oligodendroglial cells in the white matter pass through an active metabolic stage during myelination, in which they are highly radio-

sensitive (fig. 53, 54). The influence of early postnatal X-irradiation on celldifferentiation was studied in the second series of experiments. Under these circumstances the peripheral part of the dendritic tree of Purkinjecells did not develop (cf. figs. 68 and 69), which may be related to absence of parallel fibres. The nucleus and cytoplasm of these cells however developed in a completely normal way. In these cerebella, irradiated at 2 days, many abnormal axons are present, some showing swelling, others containing honeycomb-like structures (EBELS and BLAAUW, 1968). The high radiosensitivity of glial cells in the white matter in the stage of active myelination has already been mentioned, and is in agreement with findings by GILMORE (1963) and RODGERS (1965).

c. FORMATION OF SYNAPSES

Formation of synapses takes place gradually and is paralleled by increase in enzyme histochemical activity of dehydrogenases, ATPase and AMPase in the molecular layer, and of N.ASD.E, AcPase and AChE also, in the glomeruli. The possible significance of AMPase and AChE in connexion with synaptic function is discussed but definite conclusions cannot be drawn. Different opinions exist on the morphogenesis of synaptic structures (BUNGE and BUNGE, 1965; WECHSLER, 1967; OCHI, 1967); to settle the controversies three-dimensional reconstruction is required. The presence of synapses in temporary lateral cytoplasmic processes of Purkinjecells make it improbable for these processes to be resolved later on, as suggested by CAJAL (1911), PURPURA et al. (1964), LARRAMENDI (1965, 1966); we think it more likely that in later stages these expulsions will be remodelled into proximal dendrites, which would be consistent with CAJAL's (1911, pp. 100 seqq.) interpretation of the development of climbing fibres.

d. DEVELOPMENT OF BLOODVESSELS

The development of bloodvessels was studied enzyme histochemically. An over-all correlation exists between development of bloodvessels and of metabolic activity as seen histochemically. For some enzymes (AlkPase, ATPase, ChE) a function in the blood-brain barrier complex has been postulated. In view of the striking differ-

ences between these enzymes, this cannot equally hold for all of them. Also, the species-variation for AlkPase and ChE has, as far we know, no counterpart in specific differences in blood-brain barrier properties.

Chapter VII: EVALUATION

In this chapter the methods of investigation used in this study are evaluated.

a. ENZYMEHISTOCHEMISTRY

Enzymehistochemistry shows many different features from those seen with other histological methods. The increasing metabolic activity of various structures can be much more directly visualized. Changes in cytoplasmic enzyme activity can be interpreted as signs of celldifferentiation. Some celltypes show enzyme activity by which these cells can be identified. Bergmann glialcells can be identified in early stages of development by their high α NE activity (fig. 29), in later stages and in irradiated cerebella by high G6PDH and ICDH activity (figs. 64 and 65). Golgicells can be distinguished from Purkinjecells in cerebella after irradiation at 2 days by means of their relatively high AcPase content (fig. 71). In our material dehydrogenase and TPPase activity could be correlated with the presence of mitochondria and Golgi complexes respectively; no such correlation could be established between AcPase activity and the presence of lysosomes. It is suggested (cf. NOVIKOFF, 1967) that AcPase activity in immature Purkinjecells may be located in vesicles ("primary lysosomes") or even in endoplasmic reticulum. Many difficulties arise when an attempt is made more directly to correlate results of histochemical and biochemical investigation. Also, the significance of many enzymes demonstrated histochemically has not yet been established. Striking species-variations as to enzyme activity in otherwise identical structures mar efforts to establish correlation between enzyme activity and other features (morphological, physiological, pharmacological).

b. ELECTRONMICROSCOPY

Advantages and disadvantages of electronmicroscopy for the investigation of developing nervous tissue are obvious. The problem of *artifacts* is discussed for two findings. The occurrence of "**dark neurones**" (also seen lightmicroscopically) is considered by some investigators to be an artifact, while others hold it to be the expression of *in vivo*-existing properties of these cells. We feel that, by special procedures, it might be possible to avoid these dark neurones. But this does not exclude that the possibility of their occurrence has some physiological substratum. This opinion is strengthened by our finding that dark Purkinjecells never occur in young cerebella (<12 days), regardless of the way in which the material was processed (immersion- or perfusion-fixation).

A second problem in which the possibility and significance of artifact looms large is the size of the **extracellular space**. In our material this space decreases in size during development, whereas the compartment occupied by clear glial cellprocesses increases. This finding is in agreement with that of many other authors (VERNAKIS and WOODBURY, 1965; KARLSSON, 1967; PYSH, 1967; CALEY and MAXWELL, 1968; DEL CERRO and SNIDER, 1968a). But we think that precaution is required in extrapolating these morphological aspects into the *in vivo*-situation. Especially because, due to changes in water content (e.g., BLINDERMAN and BROWN, 1966) and osmolality (MEDDA et al., 1966) of brain tissue during development, the relation itself between the *in vivo*-situation and the electronmicroscopical "Aequivalent-Bild" might alter.

c. IRRADIATION

It is shown that the conditions to be satisfied by experimental approaches in order to yield results significant for the normal development, are in fact fulfilled: the effect is rapid, reproducible and selective, and no unspecific tissue reaction ensues from irradiation. Also, some results not yet reported are discussed. After early post-natal irradiation a significant diminution of body growth is seen (vide p. 90; cf. MOSIER and JANSON, 1967). Further, the resemblance is stressed between the effect of irradiation and cerebellar atrophy mainly affecting the granular layer ("cerebellopetal atro-

phy" of Bielschowsky) as seen in other conditions: as a primary type (NORMAN, 1940; GOULON et al., 1968), in lipidoses, in congenital ataxia of animals (KILHAM and MARGOLIS, 1966a, b; KILHAM et al., 1967) and in mutant mice (SAX et al., 1968). The possibility to build different types of abnormal cerebellum might give rise to interesting physiological and pharmacological investigation. Radiobiological aspects, such as the cause of dissimilarity in radiosensitivity between different cells and cells in various stages of development, were neither investigated nor discussed in this study.

Chapter VIII: EPILOGUE

In the last chapter an excursion is made into the field of growth and differentiation of the central nervous system in general. Our knowledge of the mechanisms regulating the development of the nervous system is still meagre. We do not know how cellproliferation is regulated, why a certain cell escapes from the recurrent cycle of proliferation and starts differentiating into a cell of a certain type. It is still unknown how migratory movements are coordinated, what induces the formation of synaptic contact and what regulates the development of the cerebellar vascular pattern.

Some factors influencing the development of the nervous system, such as hormones, nutrition, "peripheral load" (HAMBURGER and KEEFE, 1944; HAMBURGER and LEVI-MONTALCINI, 1949, 1955), environment and experience, are briefly discussed. The dilemma of priority of form or function is raised. Wider importance of assessing the relative significance of these factors becomes obvious when they are discussed in the context of developmental psychology and pedagogy.

